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Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1)

(cytotoxicity/immunofluorescence/radioimmunoassay/preimplantation mouse embryo/teratocarcinoma stem cells)

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ABSTRACT **A monoclonal antibody derived by fusion of mouse myeloma cells with spleen cells from a mouse immunized with F9 teratocarcinoma cells is described. This antibody, which reacts with embryonal carcinoma cells of mouse and human origin and with some preimplantation stage mouse embryos, defines an embryonic stage-specific antigen. This stage-specific antigen (SSEA-1) is first detected on blastomeres of 8-cell stage embryos. Trophectodermal cells are transitorily positive; however, each cell in the inner cell mass eventually expresses this antigen.**

The importance of embryonic stage-specific molecules in the regulation of cell interactions and cell sorting during development and differentiation has been postulated; however, experimental confirmation of this postulate is scarce. Several candidates for stage-specific molecules have been found by using antisera, raised by both syngeneic (1, 2) and xenogeneic (3) immunization with embryonal carcinoma cells (ECC) or by xenogeneic immunization (4) with mouse embryos. Indeed, inhibition of development of preimplantation mouse embryos by Fab fragments isolated from antisera to the murine teratocarcinoma cell F9 has recently been reported (5). However, such sera contain antibodies to multiple antigenic determinants, which precludes precise definition of embryo stage-specific antigens. In order to circumvent this difficulty and to study the stage-specific molecules in a methodical fashion, we are producing monoclonal antibodies (6) reactive with teratocarcinoma cells and embryos. Production and characterization of one such antibody is described here.

MATERIALS AND METHODS

Preparation of Monoclonal Reagents. BALB/c mice were immunized by weekly intraperitoneal injection of 10^7 irradiated F9 cells. The mice were tail-bled 7 days after each injection, and sera were tested for reactivity on F9 cells. Three days after the seventh immunization, the spleen was removed from one mouse, which showed a high titer of antibody reactivity. Splenic lymphocytes were isolated and fused with the P3-X63-Ag8 mouse myeloma cell line as described (6–8). Hybrid cell lines were isolated by growth of the fusion mixtures in Dulbecco's modification of Eagle's minimal essential medium containing 10% fetal bovine serum, 2 mM glutamine, and the HAT components (hypoxanthine/aminopterin/thymidine) (9). Supernatants from Linbro wells containing growing colonies were tested for reactivity on F9 cells by an indirect antibody binding radioimmunoassay (RIA). One positive colony was transferred to mass culture and cloned. Supernatants from clones were also tested in RIA and the positive clones were maintained in tissue culture. Supernatants were collected from dense cultures of specific antibody-producing hybrids, clarified by centrifugation, pooled, and stored at −70°C until further use. BALB/c mice were injected intraperitoneally with 10^6 specific antibody-producing hybrid cells mixed with Freund's complete adjuvant. Mice with visible ascites were anesthetized and bled by cardiac puncture, and ascites fluid was removed. Sera and ascites fluid were clarified by centrifugation and frozen at −70°C until further use.

Cell Lines and Maintenance. For characterization of antibody reactivity, the cell lines listed in Table 1 were used. These cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 15% heat-inactivated fetal bovine serum without the addition of antibiotics.

Embryos. Unfertilized eggs and preimplantation embryos were isolated from random-bred ICR mice as previously described (18). Zonae pellucidae were removed by Pronase digestion and the embryos were left for 4 hr in Whitten's medium before testing. Inner cell masses were isolated from blastocysts by immunosurgery (19).

Reagents. Immunoglobulin (Ig) class-specific reagents (goat IgG anti-mouse μ, γ_1, γ_{2A}, γ_3, κ, and λ chains) were purchased from Bionetics, Inc. Goat IgG anti-mouse IgM (heavy chain-specific) conjugated with fluorescein isothiocyanate (GAM IgM FITC) and goat IgG anti-mouse IgG (heavy and light chain) (GAMIG) were purchased from Cappell Laboratories, Cochranville, PA. Serum from a rabbit injected with MOPC 104E tumor (Bionetics, Inc.) was utilized as an anti-μ chain-specific reagent after passage through a mouse IgG immunoabsorbant column (RAMμ). Specificity of all reagents was tested by the Ouchterlony immunodiffusion technique. Reagents were iodinated by the chloramine-T method (20). Rabbit and guinea pig serum were collected, aliquoted, and stored at −70°C until use as a complement source; these sera were screened for spontaneous cytotoxicity on teratocarcinoma cells and when necessary absorbed (21) with F9 cells.

Techniques. For all serologic tests, cells were harvested by a brief exposure to 0.5% trypsin in 0.1 M EDTA and pipetted to obtain single cell suspensions in 0.01 M Hepes-buffered Eagle's minimal essential medium containing 10% fetal bovine serum. Absorptions were performed either two times with equal volumes of packed cells and diluted antiserum or for quantitative absorption once with known numbers of cells at a constant dilution of antiserum (80% of its maximum binding activity). For all serologic tests, embryos were exposed in 20-μl droplets to increasing dilutions of antiserum in Hepes-buffered Whitten's medium (HWM) (22), washed three times in the same medium, and then transferred to guinea pig complement (1:16 in HWM), GAM IgM FITC (1:10 in HWM), or RAMμ labeled with ^{125}I (50,000 input cpm in Dulbecco's modified phosphate-buffered saline with 5% FBS and 0.02% sodium azide)

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Abbreviations: SSEA-1, stage-specific embryonic antigen 1; ECC, embryonal carcinoma cells; RIA, radioimmunoassay; SV40, simian virus 40; HWM, Hepes-buffered Whitten's medium.

Table 1. Cell lines and their origins

Cell line	Species	Characteristics	Origin	SSEA-1*
F9	Nullipotent ECC (10)	129/Sv mouse	OTT6050 tumor	11,000
PCC4	Pluripotent ECC (11)	129/Sv mouse	OTT6050 tumor	10,600
ND-1	Pluripotent ECC (11)	129/Sv mouse	OTT6050 tumor	9,400
SCC1	Nullipotent ECC (12)	129/Sv mouse	402-A-II tumor	9,600
NG2	Pluripotent ECC (13)	129/Sv mouse	OTT5584 tumor	10,300
LT/SV	Pluripotent ECC	LT mouse	LT tumor	12,500
MH-15	Nullipotent ECC	BALB/c J mouse	MH-15 tumor	10,700
FA-25	Nullipotent ECC	AKR/J mouse	FA-25 tumor	10,400
PYS-2	Parietal yolk sac (14)	129/Sv mouse	OTT6050 tumor	0
OTT6050f	Fibroblasts	129/Sv mouse	OTT6050 tumor	0
Tera 1	Mixed cultures (15) containing ECC	Human	Lung teratocarcinoma	4,000
Tera 2	Mixed cultures (15) containing ECC	Human	Lung teratocarcinoma	3,000
Tera 2f	Fibroblasts	Human	Lung teratocarcinoma	200
B3T3SV	SV40-transformed fibroblasts	BALB/c mouse	Embryo fibroblasts	0
C57SV	SV40-transformed fibroblasts (16)	C57BL/6J mouse	Embryo fibroblasts	0
K129SV	SV40-transformed fibroblasts (16)	129/J mouse	Adult kidney	0
KG_{IX}-SV	SV40-transformed fibroblasts (16)	129/J mouse	Adult kidney	0
KCA	Adenovirus-5-transformed fibroblasts	C57BL/6J mouse	Adult kidney	0
OAIB	Adenovirus-2-transformed ependymoma	C3H/He mouse	Adult brain	0
BW5147	T-cell lymphoma	AKR mouse	Thymoma	0
LNSV	SV40-transformed fibroblasts (17)	Human	Skin fibroblasts	700

SV40, simian virus 40.

* Stage-specific embryonic antigen 1 measured as cpm bound in indirect ^{125}I RIA using serum from hybridoma-bearing mouse (dilution 1:32,000). Background 400–600 cpm is subtracted. For details see legend to Fig. 1.

and incubated for 1 hr at 37°C. Cytotoxicity was scored as previously described (18). After six washes in HWM, embryos were transferred to a small drop of the same medium under paraffin oil on a microscope slide and examined with a Leitz microscope equipped with fluorescent epiillumination and ×50 water immersion lens. For RIA, embryos were washed six times in phosphate-buffered saline/fetal bovine serum/azide, transferred to tubes, and assayed for radioactivity in a Packard gamma counter. RIA and antibody-mediated complement-dependent lysis on tissue-cultured cells were performed as previously described (23).

RESULTS

Detection and Isolation of Antibody-Producing Hybrid Cells. Supernatant fluids from 14 separate colonies of cells proliferating in hypoxanthine/aminopterin/thymidine medium were tested in RIA on target F9 cells with ^{125}I-labeled GAMIG as the second reagent. Supernatant from one of these colonies was found positive. This colony was propagated *in vitro* and cloned by limiting dilution.

Characterization of Antibody. Supernatant fluids from the parental colony and a clone were tested with mouse immunoglobulin class-specific reagents in Ouchterlony double immunodiffusion. Precipitation lines of identity were found with goat IgG anti-mouse γ_1 (secreted by the parental myeloma), with goat IgG anti-mouse μ chain sera, and with goat IgG anti-mouse κ; no activity with the anti-mouse λ chain sera was found. Sodium dodecyl sulfate gel electrophoresis of reduced immunoprecipitates (using RAMμ) of radiolabeled supernatants from the hybrid colony showed a Coomassie blue-stained band of 68,000 M_r, compatible with the molecular weight of mouse μ chain. After these determinations were made, anti-mouse μ chain-specific reagents were used in all indirect immunologic testing.

Sera and ascites fluid from hybrid tumor-bearing mice were titered on F9 teratocarcinoma target cells. In the interests of uniformity the serum from one of these mice has been used for the remainder of the work reported herein.

Reactivity of the Monoclonal Reagent on Cell Lines. In order to determine specificity of the monoclonal reagent, it was reacted on a series of mouse and human cell lines in RIA (Table 1 and ref. 24). The reagent was titered on F9 target cells and the remaining cells were tested on the plateau of the titration curve (1:8000) and with two dilutions (1:32,000 and 1:128,000) in the descending portion of curve. The reactive cells include each mouse teratocarcinoma stem cell line tested, and two human teratocarcinoma-derived cell lines. Nonreactive cells include several nonstem cell lines derived from mouse and human teratocarcinoma and several mouse transformed cell lines derived from different inbred mouse strains.

These results were corroborated by absorption of the monoclonal reagent with the same cell lines. When quantitative absorption was performed with increasing numbers of cells at 1:32,000 dilution (approximately 75% of maximum binding on F9 cells) and the absorbed reagent was tested on F9 cells, the cell lines again fell into the same distinct categories (Fig. 1). Each of the mouse teratocarcinoma stem cell lines tested absorbed the antiserum reactivity; MH was most effective (5.5×10^4 cells removed 50% of the binding activity on F9 cells) and

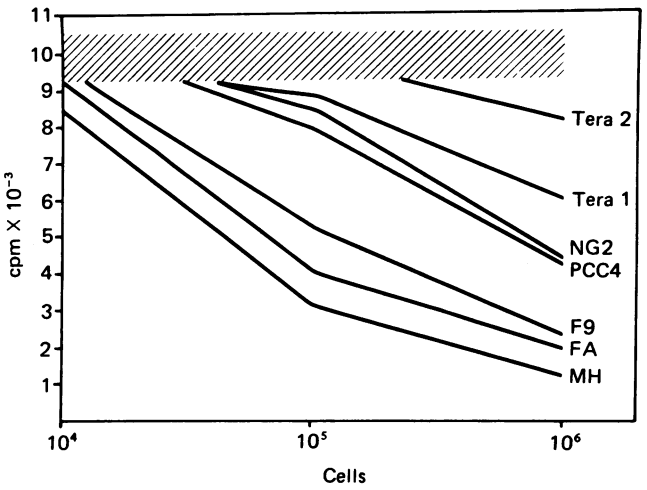

FIG. 1. Absorption analysis of antibody reactivity by RIA on F9 target cells. Aliquots (200 μl) of serum diluted 1:32,000 were absorbed with 10^6, 10^5, and 10^4 of each of the cell lines (4°C, 1 hr). The absorbed sera were clarified by centrifugation and tested in indirect RIA in triplicate on F9 target cells. Triplicate replicas of 5×10^5 cells were incubated with 100 μl of diluted supernatant (4°C, 1 hr); the final serum dilution was 1:64,000 (50 μl absorbed serum added to 50 μl containing 5×10^5 cells). The cells were washed three times in phosphate-buffered saline/5% fetal bovine serum/0.02% azide and then incubated with 100 μl of ^{125}I-labeled RAMμ diluted to give 50,000 input cpm. After an additional three washes, the cells were transferred to tubes for gamma counting. As a control, F9 was incubated with the same dilutions of normal BALB/c serum and the average cpm (400–600) was subtracted from the total cpm bound to give specific cpm. Cells that did not absorb any activity (shaded area) include K129SV, KG_{IX}-SV, B3T3SV, C57SV, KCA, OAIB, OTT6050f, PYS, LNSV, BW5147, and Tera 2f.

NG-2 was the least active (8×10^5 cells were required to remove the same amount of antiserum reactivity). With the human teratocarcinoma cell lines, 40% of the reactivity was removed by 10^6 Tera 1 cells, and 10^6 Tera 2 cells removed 20% of the binding activity on F9 cells. The binding activity of supernatant from the hybridoma was also tested after a single absorption at 1:10 dilution with 1.2×10^8 epididymal mouse sperm (data not shown), and it no longer reacted with F9 target cells. When volumes of packed cells equivalent to the packed cell volume of 10^6 F9 cells were used to absorb 200 μl of 1:32,000 diluted antisera human sperm, 129 strain mouse kidney and brain removed 80% of the binding ability of the monoclonal reagent on F9 cells while an equivalent volume of mouse spleen cells did not affect the binding.

Culture fluid from growing hybridomas and serum from hybridoma-bearing mice were titered in complement dependent lysis. The dilution of supernatant from the antibody-producing hybrid cells necessary to lyse 50% of F9 cells was 1:50, while serum from hybrid tumor-bearing mice lysed 75% of F9 cells at 1:800,000 dilution (Table 2). Other embryo-derived murine teratocarcinoma cells were also lysed by this serum and complement with high efficiency. A small percentage of the Tera 1 human teratocarcinoma-derived cell lines were lysed at low dilutions of serum. The human teratocarcinoma cell line Tera 2 and control mouse and human cell lines were not lysed (Table 2).

Reactivity of the Monoclonal Reagent with Preimplantation Mouse Embryos. Embryos were tested with supernatant fluids from the hybridoma culture and with serum from hybrid tumor-bearing mice in RIA. No antibody was bound to unfertilized eggs, zygotes, and 2- and 4-cell stage embryos. Late 8-cell stage embryos and morulae bound antibody with increasing efficiency, and the amount of binding was decreased on blastocysts. An increase in the amount of antibody bound was seen on inner cell masses, both freshly isolated and grown in culture, compared with blastocysts (Fig. 2).

Table 2. Complement-mediated antibody-dependent cytolysis

Dilution of antiserum to give 50% lysis	Cell lines
>1:800,000	F9, MH, SCCl
1:140,000	ND-1
1:100,000	PCC3, PCC4
<1:100	PYS, B3T3SV, OAIB, Tera 2f, Tera 1*

* At dilutions up to 1:10,000, approximately 20% specific lysis was observed.

These same embryonic stages were tested in complement-dependent lysis. No lysis of embryos prior to the 8-cell stage was observed. Ten to 20% of 8-cell stage embryos were lysed with antiserum dilutions up to 128,000. Morulae, blastocysts, and inner cell masses were lysed with high efficiency (Table 3).

Similar results were obtained with embryos in indirect immunofluorescence assays; unfertilized eggs, zygotes, and 2- and 4-cell stage embryos were negative. Of the 8-cell stage embryos investigated, approximately 10% consisted of all positive blastomeres, 10–20% had some positive and some negative blas-

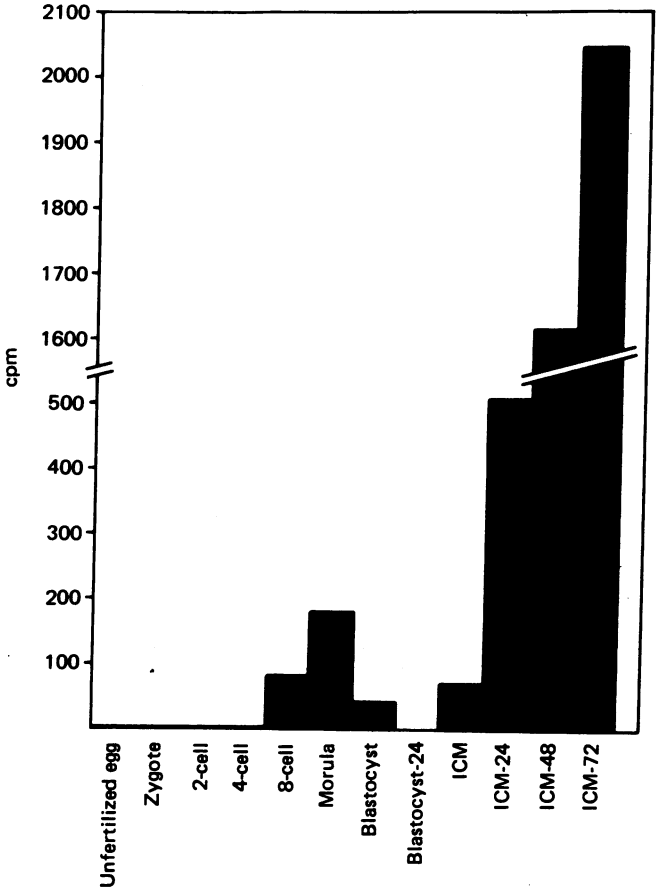

FIG. 2. Indirect ^{125}I-binding assay of serum from hybridoma-bearing mouse to preimplantation stage mouse embryos. Ten embryos of each stage were incubated with antiserum diluted 1:100 or normal BALB/c serum (1:10) as a control. The graph represents the average value from five separate experiments. ICM, inner cell masses. Numbers after development stages denote hours of *in vitro* cultivation prior to analysis.

Table 3. Complement-mediated lysis of preimplantation mouse embryos by monoclonal antiserum*

Developmental stage	Dilution of antiserum to give 50% lysis
Unfertilized egg	<1:5†
Zygote	
2-cell stage	
4-cell stage	
8-cell stage	<1:5‡
Morula	1:256,000§
Blastocyst	1:64,000§
Inner cell masses	1:64,000§

* Cumulative data of six independent experiments; 10 embryos used for each dilution of antiserum in each experiment.
† Lysis was not observed at any dilution.
‡ Lysis of 10–20% of embryos was observed at end dilution up to 1:128,000.
§ Even at lowest dilutions, 10–30% of embryos were not lysed.

tomeres, and the remaining embryos were totally negative. The difference between positive and negative blastomeres was easily discernible (Fig. 3) with antiserum dilutions of 1:100. At later developmental stages (morulae, blastocysts, inner cell masses), the number of completely and partially positive embryos increased significantly (70–90%); however, throughout the range of antiserum dilutions there were always some totally negative embryos. Inner cell masses grown *in vitro* for up to 3 days were positive, although some negative cells were also present. Ectoderm exposed by removing the outer layer of entoderm from inner cell masses grown *in vitro* (25) was always completely positive (Fig. 3*g*).

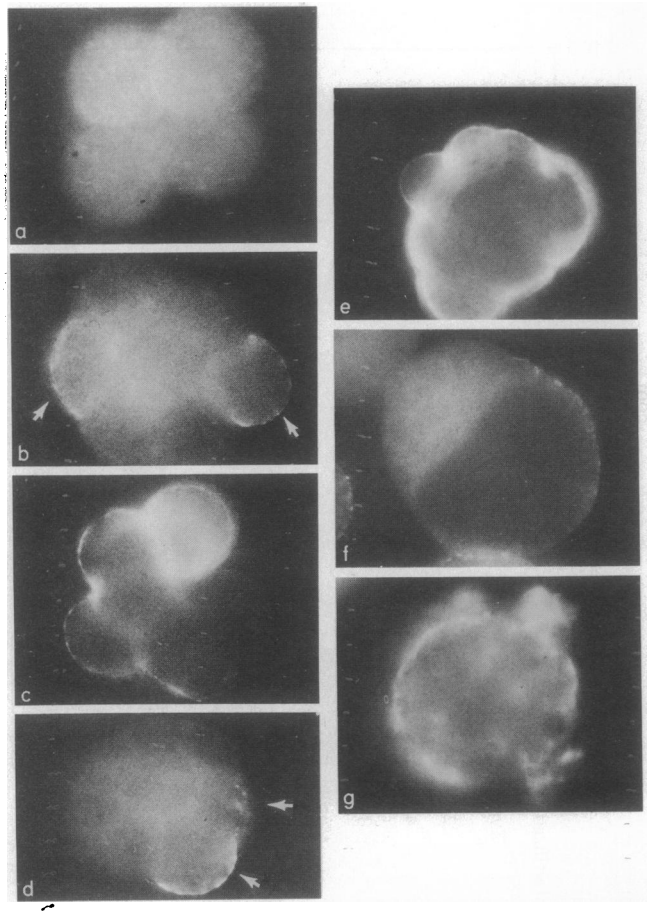

FIG. 3. Preimplantion mouse embryos were exposed to serum from a hybridoma-bearing mouse for 1 hr, washed, and exposed to GAM IgM-FITC for 1 hr. (×300.) (*a*) Four-cell stage; (*b*) 8-cell stage, two positive blastomeres (arrows); (*c*) 8-cell stage, four positive blastomeres; (*d*) morula, two positive blastomeres (arrows); (*e*) morula, completely positive; (*f*) blastocyst, weakly positive mural trophectoderm; (*g*) ectoderm from inner cell mass grown *in vitro* for 3 days—all cells positive.

DISCUSSION

The monoclonal antibody described here is specific for an antigenic determinant present on teratocarcinoma stem cells, be they of human or mouse origin. All other differentiated cell lines tested were negative, including differentiated cell lines derived from the same tumors as the teratocarcinoma stem cell lines. Because some of the mouse and human teratocarcinoma cell lines are mixed cultures containing both differentiated and undifferentiated cells, it is possible by varying the culture conditions to greatly decrease or completely eliminate stem cells from some cultures as judged by morphological criteria. Such depleted cultures reacted very poorly or not at all with monoclonal antibody (data not shown) in RIA and in immunofluorescence.

Appearance of antigen at late 8-cell stage and its disappearance from trophectoderm might indicate the beginning and the cessation of synthesis or the uncovering and covering of the existing antigen. However, the reason for the unequal distribution of stage-specific embryonic antigen 1 (SSEA-1) seen on preimplantation embryos is not clear. Because no consistent pattern of positive cells could be discerned, it is unlikely that the antigen represents a marker of any cell lineage unless we assume that cells with different developmental fate are randomly distributed in mouse morulae. The other possibility is that antigen is expressed differently during the cell cycle, as has been observed with other antigens.

SSEA-1 is present in most but not all cells of freshly isolated inner cell masses. When inner cell masses are grown *in vitro*, they develop a layer of entodermal cells on the outside (19), and this layer is mostly positive after 48 hr of culture and remains so for all the time we were able to examine it (120 hr). The entodermal layer can be selectively removed by repeated immunosurgery (25), and thus the exposed ectodermal layer was uniformly positive throughout the entire culture period (24–120 hr).

Several conventional antisera raised by syngeneic and xenogeneic immunization against teratocarcinoma stem cells and preimplantation mouse embryos have been described (for review see refs. 1, 3, 26, 27). In an attempt to compare our monoclonal reagents with these previously described antisera, we have to bear in mind that the monoclonal antibody might represent a part of the activity of any of the conventional antiserum. This may be the case when a conventional antiserum reacts with the same cells and preimplantation stage embryos as the monoclonal reagent, even though the conventional antiserum may react with additional cells. However, if the monoclonal reagent reacts with cells or embryonic stages with which a conventional antiserum does not react, then we can conclude that monoclonal reagent is not part of activity in the conventional antiserum. On the basis of these premises, antisera to the F9 antigen (28) do not contain reactivity to the same antigenic specificity as the monoclonal reagent because the activity of F9 antiserum is not removed by absorption with brain and kidney. On the other hand, absorption with tissue homogenates is difficult to control and we cannot exclude the possibility of nonspecific absorption with brain and kidney. The antiteratocarcinoma serum described by Stern *et al.* (2) has a range of activity very similar to that of our monoclonal antibody and its activity is also absorbed with mouse brain and kidney.

Similarly, antiserum against antigen I (3) defined by rabbit antiserum to mouse teratocarcinoma cells possessed, in addition to other activities, all the activities displayed by this monoclonal antibody. Additionally, in parallel with our results, antigen I antiserum does not react with trophectodermal cells of the late, hatched blastocyst.

The monoclonal antibody described herein can thus be a part of several described antisera, and confirmation of this must await the molecular identification of the antigenic specificity. Our preliminary data from immunoprecipitation of extracts of ^{125}I-labeled F9 cells and sodium dodecyl sulfate gel electrophoresis indicate that SSEA-1 might be a glycolipid because specifically precipitated material runs in front of bromophenol blue marker and is extractable with chloroform/methanol.

During preparation of this manuscript we became aware of recent results obtained by Stern *et al.* (29) and Willison and Stern (30). They hybridized mouse myeloma cells with spleen cells from rats immunized with mouse spleens and isolated antibody-producing hybrids. One monoclonal antibody reacted with mouse teratocarcinoma cells and sheep erythrocytes. Detailed analysis of this antibody indicated that its reactivity pattern coincides with the distribution of Forssman antigen. The reactivity of this antiserum on teratocarcinoma cells and preimplantation mouse embryos closely resembles the activity of our monoclonal antibody. We, therefore, tested our monoclonal antibody against sheep and ox erythrocytes, using hemagglutination, hemolysis, and RIA as well as absorption and testing of absorbed serum on F9 cells (data not shown). No reactivity of the monoclonal antibody to SSEA-1 was found with sheep erythrocytes in any direct tests and absorption with both sheep and ox erythrocytes removed the same minimal amount of activity. We can conclude that SSEA-1 is different from Forssman antigen, though it appears that both might be present on the same cells and embryonic stages. Because the Forssman antigen is also a glycolipid, it is possible that several glycosyltransferases are synthesized or activated during early preimplantation development and their products are then presented on the embryonic cell surface. SSEA-1 begins to be expressed on 8-cell stage embryos and the Forssman antigen on late morulae. Concordant biochemical analysis of the antigenic specificities and reactivity patterns on early mouse embryos should elucidate some of the cell surface changes during early development.

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